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FORM PTO-1390 (REV. 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
0760-0305P	
U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	
10/089695 NEW	

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/05214	August 3, 2000	----

TITLE OF INVENTION
METHOD FOR PROMOTING EFFICIENCY OF GENE TRANSFER INTO PLANT CELLS

APPLICANT(S) FOR DO/EO/US
HIEI, Yukoh; KASAOKA, Keisuke; ISHIDA, Yuji

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39 (1).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau. WO 02/12521
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   ✔a. ☒ is transmitted herewith.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4)
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 20. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98, Form PTO-1449(s), and International Search Report (PCT/ISA/210) with 0 cited document(s).
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information:
    ✔1.) PCT/IB/308
    ✔2.) Four (4) sheets of Formal Drawings

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JC13 Rec'd PCT/PTO 03 APR 2002

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
NEW 10/089695	PCT/JP00/05214	0760-0305P

	CALCULATIONS PTO USE ONLY	
21. ☒ The following fees are submitted:		
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. **$1,040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO. **$740.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4). **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☒ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	48 - 20 =	28	X $18.00	$ 504.00	
Independent Claims	1 - 3 =	0	X $84.00	$ 0	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)		Yes	+ $280.00	$ 280.00	
			TOTAL OF ABOVE CALCULATIONS =	$ 1804.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$ 0	
			SUBTOTAL =	$ 1804.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$ 0	
			TOTAL NATIONAL FEE =	$ 1804.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ 0	
			TOTAL FEES ENCLOSED =	$ 1804.00	
			Amount to be: refunded	$	
			charged	$	

a. ☒ A check in the amount of $ **1804.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account. No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-2448.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

Send all correspondence to:
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Date: April 3, 2002

By _____
Gerald M. Murphy, Jr., #28,977

/cqc

PATENT
0760-0305P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant:	HIEI, Yukoh et al.		
Int'l. Appl. No.:	PCT/JP00/05214		
Appl. No.:	New	Group:	
Filed:	April 3, 2002	Examiner:	
For:	METHOD FOR PROMOTING EFFICIENCY OF GENE TRANSFER INTO PLANT CELLS		

PRELIMINARY AMENDMENT

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, DC 20231

April 3, 2002

Sir:

The following Preliminary Amendments and Remarks are respectfully submitted in connection with the above-identified application.

AMENDMENTS

IN THE SPECIFICATION:

Please amend the specification as follows:

Before line 1, insert --This application is the national phase under 35 U.S.C. § 371 of PCT International Application No. PCT/JP00/05214 which has an International filing date of August 3, 2000, which designated the United States of America.--

Docket No. 0760-0305P

IN THE CLAIMS:

Please amend the claims as follows:

6. (Amended) The method according to claim 1 or 2, wherein the heat treatment is carried out for 5 seconds to 24 hours.

8. (Amended) The method according to claim 1 or 2, wherein the centrifugation is carried out under a centrifugal acceleration of 100G to 250,000G.

11. (Amended) The method according to claim 1 or 2, wherein said centrifugation acceleration of 1000G to 150,000G.

12. (Amended) A method for preparing a plant characterized by using the method according to claim 1 or 2.

13. (Amended) Plant cells, plant tissue or plant prepared by the method according to claim 1 or 2.

14. (Amended) The method according to claim 1 or 2, wherein said plant cells or plant tissue used are(is) originated from an angiosperm.

16. (Amended) Angiosperm cells, angiosperm tissue or angiosperm prepared by the method according to claim 14.

19. (Amended) The monocotyledon cells, monocotyledon tissue or monocotyledon prepared by the method according to claim 17.

22. (Amended) The cells of the plant belonging to family Gramineae, the tissue of the plant belonging to family Gramineae, or the plant belonging to family Gramineae prepared by the method according to claim 20.

25. (Amended) Rice cells, rice tissue, rice, maize cells, maize tissue or maize prepared by the method according to claim 23.

Docket No. 0760-0305P

REMARKS

The specification has been amended to provide a cross-reference to the previously filed International Application.

The amendment to the claims is merely to correct improper multiple dependencies and to place the application into better form for examination. Entry of the above amendments is earnestly solicited. An early and favorable first action on the merits is earnestly solicited.

Attached hereto is a marked-up version of the changes made to the application by this Amendment.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By _____
Gerald M. Murphy, Jr., #28,977

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GMM/cqc
0760-0305P

Attachment: VERSION WITH MARKINGS TO SHOW CHANGES MADE

(Rev. 02/21/02)

Docket No. 0760-0305P

VERSION WITH MARKINGS TO SHOW CHANGES MADE

The claims have been amended as follows:

6. (Amended) The method according to [any one of claims 1 to5]claim 1 or 2, wherein the heat treatment is carried out for 5 seconds to 24 hours.

8. (Amended) The method according to [any one of claims 1to 7]claim 1 or 2, wherein the centrifugation is carried out under a centrifugal acceleration of 100G to 250,000G.

11. (Amended) The method according to [any one of claims 1 to 10]claim 1 or 2, wherein said centrifugation acceleration of 1000G to 150,000G.

12. (Amended) A method for preparing a plant characterized by using the method according to [claim 1 to 11]claim 1 or 2.

13. (Amended) Plant cells, plant tissue or plant prepared by the method according to [claims 1 to 12]claim 1 or 2.

14. (Amended) The method according to [any one of claims 1 to 11]claim 1 or 2, wherein said plant cells or plant tissue used are(is) originated from an angiosperm.

16. (Amended) Angiosperm cells, angiosperm tissue or angiosperm prepared by the method according to claim 14[or 15].

19. (Amended) The monocotyledon cells, monocotyledon tissue or monocotyledon prepared by the method according to claim 17[or 18].

22. (Amended) The cells of the plant belonging to family Gramineae, the tissue of the plant belonging to family Gramineae, or the plant belonging to family Gramineae prepared by the method according to claim 20[or 21].

25. (Amended) Rice cells, rice tissue, rice, maize cells, maize tissue or maize prepared by the method according to claim 23[or 24].

4/prts

SPECIFICATION

Method for Promoting Efficiency of Gene Transfer into Plant Cells

Technical Field

The present invention relates to a method for promoting efficiency of gene transfer into plant cells.

Background Art

The method for transformation using *Agrobacterium* has a number of excellent features including, in general, the high efficiency, the small number of copies of the transferred gene, the feature that the gene may be transferred without fragmenting a specific region called T-DNA, and the feature that the frequency of mutation occurred during cultivation is low because transformants may be obtained by cultivation for a short period of time. Therefore, the method is widely used as the most useful method for transforming various plants.

Although the *Agrobacterium* method is an extremely excellent method for transforming plants, whether the transformation is successful or not and the transformation efficiency largely varies depending on the plant species, genotype and the plant tissue used (Potrykus et al. 1998 (Reference (36))). That is, there are species with which the transformation has not been successful, and species with which the transformation may be attained only with limited varieties. Further, there are species with which the tissue to be used is limited so that a large amount of materials cannot be treated. To prepare a practical variety by genetic recombination(genetic engineering), it is necessary to prepare a large number of transformed plants and to select the line having the desired character therefrom. However, at present, the type of plants with which a large number of transformed plants may be prepared for this purpose is limited. Thus, to develop an improved method by which this problem may be overcome is strongly demanded.

Although the method for transformation via *Agrobacterium* differs in the

starting material, composition of the culture medium and the like, it is almost common to the *Agrobacterium* method that the method comprises making a tissue which is a starting material contact a suspension of *Agrobacterium*, selecting transformed cells after co-culturing, and growing transformed plants. The *Agrobacterium* is infected without performing a special treatment except for sterilization treatment which is carried out as required (Rogers et al. 1988 (Reference (37)), Visser 1991 (Reference (41)), McCormick 1991 (Reference (31)), Lindsey et al. 1991 (Reference (30))). Thus, studies for improving transformation system has been carried out mainly on the *Agrobacterium* strain, constitution of the vector, composition of medium, types of selection marker gene and promoter, the type of the tissue used as the material, and the like.

On the other hand, studies for changing the plant tissue before infection of *Agrobacterium* to a physiological state in which the genes are likely to be transferred have been scarcely made. If the physiological state of the tissue can be changed to such a physiological state by a simple treatment, the method is very useful, and it is expected that, in addition to the promotion of the transformation efficiency, transformation may be attained for the species or genotypes with which transformation has been hitherto difficult, that is a prominent effect. Known studies about pretreatment of plant tissue include particle gun treatment (Bidney et al., 1992 (Reference (6))) and ultrasonication treatment (Trick H. N. et al., 1997 (Reference (40))). Both of these methods aim at promoting invasion of bacteria into the plant tissue by physically injuring the tissue, so as to increase the number of plant cells infected. However, these methods are nothing more than developments of the leaf disk method (Horsch et al., 1985 (Reference (19))) and not treatments based on novel concepts. The degree of effectiveness and universality of the methods have not been clarified, and they are not used as general methods.

Disclosure of the Invention

Accordingly, an object of the present invention is to provide a method for promoting efficiency of gene transfer into plant cells, by which gene transfer can be attained simply with a higher efficiency than the conventional gene transfer by *Agrobacterium* method, without injuring the tissue.

The present inventors intensively studied to discover that in the gene transfer method using *Agrobacterium*, the gene transfer efficiency may be significantly promoted by heating and centrifuging the plant cells or plant tissue subjected to the gene transfer, thereby completing the present invention.

That is, the present invention provides a method for promoting efficiency of gene transfer into plant cells by a bacterium belonging to genus *Agrobacterium*, comprising heating and centrifuging said plant cells or plant tissue.

By the present invention, a method for promoting efficiency of gene transfer into plant cells, by which gene transfer can be attained simply with a higher efficiency than the conventional gene transfer by *Agrobacterium* method, without injuring the tissue, has been provided. The method of the present invention may be applied to both monocotyledons and dicotyledons. Further, the plants such as creeping bent grass which could not be hitherto transformed by *Agrobacterium* method can be transformed by the method of the present invention.

Brief Description of the Drawings

Fig. 1 is a drawing for showing a method for constructing pTOK233 which is an example of super-binary vectors, that may preferably be employed in the present invention.

Fig. 2 is a gene map of pNB131 which is an example of super-binary vectors, that may preferably be employed in the present invention.

Fig. 3 is a schematic view for showing the intermediate vector system and binary vector system which are major two vector systems of bacteria belonging to genus *Agrobacterium*.

Fig. 4 is a schematic view showing two binary vector systems derived from super virulent strain A281 of *Agrobacterium tumefaciens*.

In the above drawings, the following reference symbols denote the following meanings.

BL: left border sequence of T-DNA of bacteria belonging to genus *Agrobacterium*

BR: right border sequence of T-DNA of bacteria belonging to genus *Agrobacterium*

TC: tetracycline resistance gene

SP: spectinomycin resistance gene

IG: intron GUS gene

HPT: hygromycin resistance gene

NPT: kanamycin-resistance gene

K: restriction enzyme *Kpn* I site

H: restriction enzyme *Hind* III site

Amp^r: ampicillin resistance gene

BAR: bar gene

COS, cos: COS site of λ phage

ORI, ori: replication origin of ColE1

P35S: CaMV 35S promoter

Tnos: terminator of nopaline synthetase gene

virB: the *vir*B gene in the virulence region of Ti plasmid pTiBo542 contained in *Agrobacterium tumefaciens* A281

virC: the *vir*C gene in the virulence region of Ti plasmid pTiBo542 contained in *Agrobacterium tumefaciens* A281

virG: the *vir*G gene in the virulence region of Ti plasmid pTiBo542 contained in *Agrobacterium tumefaciens* A281

Vir: entire *vir* region of Ti plasmid of bacteria belonging to genus *Agrobacterium*

S Vir: entire *vir* region of Ti plasmid pTiBo542 of super virulent bacteria belonging to genus *Agrobacterium*

s vir*: fragment containing a part of *vir* region of Ti plasmid pTiBo542

Best Mode for Carrying out the Invention

The method of the present invention for promoting efficiency of gene transfer into plant cells by a bacterium belonging to genus *Agrobacterium*, comprises heating and centrifuging the plant cells or plant tissue. The plant cells or plant tissue may be contacted with the bacterium belonging to genus *Agrobacterium* under normal gravity after heating and centrifuging the plant cells or tissue, or the plant cells or tissue may be contacted with the bacterium belonging to genus *Agrobacterium* while heating and/or centrifuging the plant cells or tissue. In cases where the heat treatment and centrifugation treatment are carried out before making the plant cells or tissue contact the bacterium belonging to the genus *Agrobacterium*, these treatments may be carried out simultaneously, or one of these treatments may be carried out before the other treatment.

The conditions of the heat treatment may appropriately be selected depending on the type of the plant used and the like, and may usually be carried out at a temperature of 30°C to 60°C, preferably 33°C to 55°C, more preferably 37°C to 52°C. The time of the heat treatment may appropriately be selected depending on the heating temperature, type of the plant used, the type of the cells or tissue to be heat-treated and so on, and is usually 5 seconds to 24 hours. When the heating temperature is high, the efficiency of transferring genes may be significantly promoted even if the time of the heat treatment is short. For example, when the heating temperature is 60°C, heat treatment for about 5 seconds may significantly promote the efficiency of gene transfer. On the other hand, when the heating

temperature is as low as about 34°C, the efficiency of gene transfer may be promoted by heat treatment for several tens of hours. In most cases, particularly preferred heating conditions are 37°C to 52°C for 1 minute to 24 hours, and the appropriate heating conditions for the particular plant cells or tissue may be easily selected by a routine experiment. By heating the plant cells or plant tissue at a temperature not lower than 55°C for a long time, the plant cells may be damaged and the efficiency of transformation may be decreased. Therefore, when the heating temperature is not lower than 55°C, the heating time is preferably short, for example, not longer than 3 minutes, preferably not longer than 1 minute so as to avoid damaging of the plant cells.

The conditions for centrifugation may appropriately be selected depending on the type of the plant used and the like, and may usually be carried out under a centrifugation acceleration of 100G to 250,000G, preferably 500G to 200,000G, more preferably 1000G to 150,000G. The time for centrifugation may appropriately be selected depending on the centrifugal acceleration, type of the plant used and so on, and is usually and preferably not less than one second. There is no upper limit of the centrifugation time, and about 10 minutes may usually be sufficient for attaining the object of the centrifugation. When the centrifugal acceleration is large, the efficiency of transferring genes may be significantly promoted even if the centrifugation time is very short, for example, 1 second or less. On the other hand, when the centrifugal acceleration is small, the efficiency of transferring genes may be significantly promoted by conducting the centrifugation for a long time. In most cases, especially preferred centrifugation conditions are about 500G to 200,000G, especially 1000G to 150,000G for about 1 second to 2 hours, and the appropriate centrifugation conditions for the particular plant cells or tissue may be easily selected by a routine experiment.

The method of the present invention is characterized by using the plant cells

or plant tissue which were(was) heated and centrifuged, or by contacting the plant cells or plant tissue with a bacterium belonging to the genus *Agrobacterium* while conducting the heat treatment and/or centrifugation, and as the method for gene transfer or transformation *per se* using the bacterium belonging to the genus *Agrobacterium*, a well-known method may be applied as it is.

The method for gene transfer or transformation *per se* into plants using a bacterium belonging to the genus *Agrobacterium* is well-known in the art and is widely used.

It is known for a long time that a soil bacterium *Agrobacterium* (*Agrobacterium tumefaciens)* causes crown gall disease in a number of dicotyledons. In 1970s, it was discovered that Ti plasmid concerns the virulence, and that the T-DNA which is a part of Ti plasmid is incorporated into the plant genome. Thereafter, it was proved that the T-DNA contains genes participating in synthesis of hormones (cytokinins and auxins) required for induction of tumor, and that the genes are expressed in plants in spite of the fact that the genes are bacterial genes. A group of genes existing in the virulence region (*vir* region) in the Ti plasmid is required for the excision of T-DNA and its transfer to plants, and the border sequences existing at the both ends of the T-DNA are necessary for the T-DNA to be excised. *Agrobacterium rhizogenes* which is another bacterium belonging to the genus *Agrobacterium* has a similar system on the Ri plasmid (Figs. 3 and 4).

Since T-DNA is incorporated into the plant genome by infection of *Agrobacterium*, it was expected that a desired gene may be incorporated into the plant genome by inserting the desired gene in the T-DNA. However, since Ti plasmid is as large as not less than 190 kb, it was difficult to insert a gene into the T-DNA by a standard technique of genetic engineering. Thus, a method for transferring a foreign gene into the T-DNA was developed.

First, disarmed strains such as LBA4404 (Hoekema et al., 1983 (Reference

(14))), C58C1(pGV3850) (Zambryski et al., 1983 (Reference (44))), and GV3Ti11SE (Fraley et al., 1985 (Reference (10))), that have tumorigenic Ti plasmids from which hormone synthetase genes were eliminated, were prepared (Fig. 3). Two methods employing such a strain, that is, a method by which a desired gene is transferred into the Ti plasmid of *Agrobacterium*, and a method by which a T-DNA having a desired gene is transferred into *Agrobacterium*, were developed. One of these methods is the so called intermediate vector method (Fraley et al., 1985 (Reference (10)); Fraley et al., 1983 (Reference (11)); Zambryski et al., 1983 (Reference (44)), Japanese Laid-open Patent Application (Kokai) No. 59-140885 (EP116718)). In this method, an intermediate vector which is easy to handle by genetic manipulation techniques, in which a desired gene may be inserted, and which can be replicated in *E. coli* is transferred into the T-DNA in the disarmed type Ti plasmid of *Agrobacterium* by triparental mating (Ditta et al., 1980 (Reference (9))). Another method is the so called binary vector method (Fig. 3), which is based on the fact that although the *vir* region is necessary for the T-DNA to be incorporated into plants, it is not necessary that the T-DNA and the *vir* region exist in the same plasmid ((Hoekema et al., 1983 . Reference (14)). The *vir* region contains *vir*A, *vir*B, *vir*C, *vir*D, *vir*E and *vir*G (Plant Biotechnology Encyclopedia (Enterprise Co., Ltd. (1989)), and the *vir* region is defined as those containing all of *vir*A, *vir*B, *vir*C, *vir*D, *vir*E and *vir*G. Thus, the binary vector is a small plasmid which is replicable in both *Agrobacterium* and *E. coli*, and this plasmid is transferred into *Agrobacterium* having a disarmed type Ti plasmid. The transferred of the binary vector into *Agrobacterium* may be carried out by electroporation method, triparental mating or the like). Binary vector includes pBIN19 (Bevan, 1984 (Reference (5))), pBI121 (Jefferson, 1987 (Reference (21))), pGA482 (An et al., 1988 (Reference (2)), Japanese Laid-open Patent Application (Kokai) No. 60-70080 (EP120516)), and a number of new binary vectors have been constructed based on these vectors. In the system of Ri

plasmid, similar vectors have been constructed and are used for transformation.

Agrobacterium A281 (Watson et al., 1975 (Reference (42))) is a super-virulent strain, whose host spectrum is wide and whose efficiency of transformation is higher than other strains (Hood et al., 1987(Reference (15)); Komari, 1989 (Reference (23))). This feature is brought about by a Ti plasmid pTiBo542 contained in A281 (Hood et al., 1984 (Reference (18)); Jin et al., 1987 (Reference (22)); Komari et al., 1986 (Reference (26))).

Two new systems using pTiBo542 has been developed. One system utilizes strains EHA101 (Hood et al., 1986, Reference (17)) and EHA105 (Hood et al., 1993, Reference (16)) containing a Ti plasmid which is a disarmed type of pTiBo542. By applying these strains to the above-mentioned binary vector system, a system having a high efficiency of transformation was achieved, which is widely used for transformation of various plants. Another system is "super-binary" vector system (Hiei et al., 1994 (Reference (13)); Ishida et al., 1996 (Reference (20)); Komari et al., 1999 (Reference (28)), WO94/00977, WO95/06722) (Fig. 4). Since this system comprises a disarmed type Ti plasmid having the *vir* region (*vir*A, *vir*B, *vir*C, *vir*D, *vir*E and *vir*G) (each of these may also be hereinafter referred to as "*vir* fragment region") and a plasmid having T-DNA, this is a kind of the binary vector system. However, it is different from the binary vector in that a super-binary vector (Komari, 1990a (Reference (24))) in which a *vir* region fragment (preferably a fragment containing at least *vir*B or *vir*G, more preferably a fragment at least containing *vir*B and *vir*G) substantially lacking at least one of the fragments of *vir* region is incorporated into the plasmid having the T-DNA, i.e., the binary vector. To transfer a T-DNA region into which a desired gene has been inserted into an *Agrobacterium* having the super-binary vector, homologous recombination via the triparental mating method may be employed as an easy method (Komari et al., 1996 (Reference (27))). It has been proved that the super-binary vector gives much higher transformation

efficiency than the above-described various vector systems for a number of plant species (Hiei et al., 1994 (Reference (13)); Ishida et al., 1996 (Reference (20)); Komari, 1990b (Reference (25)); Li et al., 1996 (Reference (29)); Saito et al., 1992 (Reference (38))).

In the method of the present invention, the host bacterium belonging to the genus *Agrobacterium* is not restricted, and *Agrobacterium tumefaciens* (e.g., the above-described *Agrobacterium tumefaciens* LBA4404 (Hoekema et al., 1983 (Reference (14))) and EHA101 (Hood et al., 1986 (Reference (17))) may preferably be employed.

The method of the present invention may be applied to any of the gene transfer systems as long as it is based on the expression of the group of genes in the *vir* region in the bacterium belonging to the genus *Agrobacterium* so as to obtain significant effect. Thus, the method of the present invention may be applied to any of the vector systems such as the above-described intermediate vectors, binary vectors, super-virulent binary vectors and super-binary vectors so as to obtain the advantageous effect of the present invention. The method of the present invention may also be applied to the vector systems obtained by modification of these vectors (e.g., those wherein the entire or a part of the *vir* region of a bacterium belonging to the genus *Agrobacterium* is excised and additionally incorporated into the plasmid, or the entire or a part of the *vir* region of a bacterium belonging to the genus *Agrobacterium* is excised and is transferred into *Agrobacterium* as a part of a new plasmid). Further, needless to say, by the method of the present invention, the efficiency of transfer of the T-DNA region of wild type *Agrobacterium* is promoted so as to promote the infection efficiency.

The desired gene to be transferred into the plant may be inserted into a restriction site in the T-DNA region of the above-described plasmid by a conventional method, and the *Agrobacterium* into which the desired gene was

incorporated may be selected based on an appropriate selection marker such as a drug resistant gene against a drug such as kanamycin or paromomycin. In cases where the plasmid is large and has a number of restriction sites, it is not always easy to insert the desired DNA into the T-DNA region by an ordinary subcloning method. In such a case, the desired DNA may be inserted by the triparental mating method utilizing the homologous recombination in the cell of the bacterium belonging to the genus *Agrobacterium*.

Transfer of the plasmid into a bacterium belonging to the genus *Agrobacterium* such as *Agrobacterium tumefaciens* may be carried out by a known method including the above-mentioned triparental mating method, electroporation method, electroinjection method and chemical treatments with PEG or the like.

The gene which is to be transferred into the plant is, in principle, arranged between the left and right border sequences of the T-DNA as in the conventional method. However, since the plasmid is annular, the plasmid may contain only one border sequence. Alternatively, in cases where a plurality of genes are to be arranged at different sites, the plasmid may contain three or more border sequences. Alternatively, arrangement of the desired plasmid in the Ti or Ri plasmid may be performed in the cell of the bacterium belonging to the genus *Agrobacterium*, or the desired gene may be arranged in another plasmid. Further, the desired gene may be arranged in a plurality of types of plasmids.

Transfer of a gene into the plant cells via a bacterium belonging to the genus *Agrobacterium* may be attained by simply making the plant cells or plant tissue contact the bacterium belonging to the genus *Agrobacterium*. For example, a cell suspension of the bacterium belonging to the genus *Agrobacterium* having a population density of about 10^6 to 10^{11} cells/ml is prepared, and the plant cells or the plant tissue are(is) immersed in the suspension for about 3 to 10 minutes, followed by co-culturing the resultant on a solid medium for several days, thereby attaining the

transfer of the gene.

The cells or the tissue to be subjected to the gene transfer are(is) not restricted at all and may be a leaf, root, stem, fruit or any other portion of the plant. Further, dedifferentiated tissue such as a callus or a non-dedifferentiated tissue such as an embryo may be employed. The type of the plant is not restricted at all, and angiosperms are preferred. As long as the plant is an angiosperm, either dicotyledon or monocotyledon is preferred.

As will be concretely shown in the following Examples, by the method of the present invention, the efficiency of gene transfer is significantly promoted when compared with the conventional *Agrobacterium* method. Further, not only the efficiency of gene transfer to the plants to which genes were able to be transferred by the conventional *Agrobacterium* method, but also gene transfer can be first attained by the method of the present invention to the plants to which genes could not be hitherto transferred by the conventional *Agrobacterium* method. Therefore, the term "promoting efficiency of gene transfer" includes the cases where the gene transfer is first attained to the plants to which genes could not hitherto be transferred by a known method (that is, such a case can be considered as a case wherein the efficiency of gene transfer which was 0% by the known method is promoted).

Examples

The present invention will now be described by way of examples thereof. It should be noted that the present invention is not restricted to the following Examples.

Example 1

(1) Sample Tissue and Sample Strain

As the sample variety, Asanohikari which is a variety of Japonica rice was employed, and immature embryo was used as the material. Sample immature embryos were collected from immature seeds at 1 to 2 weeks after flowering and prepared by the method of Hiei, Y. et al (Reference (13)). That is, glumes of

immature seeds at 7 to 12 days after flowering were removed and the seeds were sterilized with 70% ethanol for 30 seconds and with 1% aqueous sodium hypochlorite solution for 10 minutes. Thereafter, immature embryos were excised and used as the samples. Calli derived from immature embryos were obtained by culturing the immature embryos on 2N6 medium (Hiei et al. 1994 (Reference (13)) (inorganic salts and vitamins of N6 (Chu C. C. 1978 (Reference (8), 1 g/l casamino acid, 2 mg/l 2,4-D) for two weeks.

As the *Agrobacterium* strains and plasmid vectors, LBA4404(pIG121Hm) (Hiei et al., 1994 (Reference (13))), LBA4404(pNB131) (see Fig. 2), and LBA4404(pTOK233) (Hiei et al., 1994 (Reference (13))) were used.

Construction of pNB131 was carried out as follows: After transferring pSB31 (Ishida Y, 1996 (Reference (20))) into *E. coli* LE392, pSB31 was transferred to *Agrobacterium* LBA4404 containing pNB1(Komari T et al., 1996 (Reference (27)) by triparental mating method (Ditta G, 1980 (Reference (9)). By homologous recombination between pNB1 and pSB1 in the cell of *Agrobacterium*, pNB131 was obtained.

The T-DNA region of pIG121Hm contain a kanamycin resistant (nptII) gene driven by nos promoter, a hygromycin resistant (hpt) gene driven by 35S promoter of cauliflower mosaic virus (CaMV), and a GUS gene driven by the 35S promoter, which GUS gene contains introns of the catalase gene of castor bean (Ohta, S. et al., 1990 (Reference (33)).

The T-DNA region of pNB131 contains a bar gene driven by 35S promoter, and a GUS gene driven by the 35S promoter, which GUS gene contains introns (described above).

The T-DNA region of pTOK233 contains an nptII gene driven by nos promoter, an hpt gene driven by 35S promoter, and a GUS gene driven by the 35S promoter, which GUS gene contains introns (described above). The plasmid

pTOK233 is a super-binary vector having high ability of transformation (Komari, T. et al., 1999 (Reference (28))).

(2) Heat Treatment

In a tube containing sterilized water, 5 to 200 mg of the sample tissue was immersed. Heat treatment was carried out by immersing the tube in a water bath of which temperature is set to a prescribed heat treatment temperature for several seconds to several tens hours. After the heat treatment, the tube was cooled with flowering water.

(3) Centrifugation Treatment

The sample tissue was placed in a centrifugal tube containing sterilized water, and centrifugation was performed at 25°C at 20,000G for 1 to 60 minutes.

(4) Infection and Co-culturing

After the heat treatment or centrifugation treatment, or combination of these treatments, the sterilized water in each tube was removed and suspension of *Agrobacterium* was added, followed by stirring the mixture with a vortex mixer for 5 to 30 seconds. Preparation of the suspension of the bacterium was carried out by Hiei Y. et al. (Reference (13)). That is, colonies of *Agrobacterium* cultured on AB medium (Chilton, M-D et al., 1974 (Reference (7))) were collected with a platinum loop and the collected bacteria were suspended in modified AA medium (AA major inorganic salts, AA amino acids and AA vitamins (Toriyama K. et al., 1985 (Reference (39)), MS minor salts (Murashige, T et al., 1962 (Reference (32)), 1.0 g/l casamino acid, 100 μM acetosyringone, 0.2 M sucrose, 0.2 M glucose). The population density of the bacterial cells in the suspension was adjusted to about 0.3 to 1 x 10^9 cfu/ml. After leaving the mixture of immature embryos and the suspension of *Agrobacterium* to stand at room temperature for about 5 minutes, the immature embryos were plated on a medium for co-culturing. As the medium for co-culturing 2N6-AS medium (Hiei et al. 1994 (Reference (13)) containing 8 g/l agarose as a

solidifier was used. The co-culturing was carried out at 25°C for 3 to 7 days in the dark, and a portion of the immature embryo was treated with X-Gluc to check the expression of the GUS gene (Hiei et al. 1994) (Reference (13)). That is, immediately after the co-culturing, the tissue was immersed in 0.1M phosphate buffer (pH 6.8) containing 0.1% Triton X-100, and was left to stand at 37°C for 1 hour. After removing *Agrobacterium* with phosphate buffer, phosphate buffer containing 1.0 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) and 20% methanol was added. After incubating the resultant at 37°C for 24 hours, tissues colored in blue were observed under microscope.

(5) Selection of Transformed Cells (Japonica rice)

After the co-culturing, the scutella of the grown embryos were divided into 4 to 7 pieces with a lancet and the divided scutella were cultured on 2N6 medium (described above) not containing a selection drug for several days under luminous condition. Then the scutella were transferred to 2N6 medium containing 50 to 100 mg/l hygromycin and cultured at 30°C under luminous condition for about 2 to 3 weeks. As the medium containing 10 mg/l phosphonothricin (PPT) as a selection drug, CC medium (Potrykus et al. 1979 (Reference (34)) containing 2 mg/l 2,4-D and not containing coconut water was used. The drug resistant calli formed on the medium were transferred to N6-7 medium (Hiei et al. 1994 (Reference (13))) and secondary selection was conducted for 7 days at 30°C under luminous condition. Each medium contained combination of 250 mg/l cefotaxime and 250 mg/l carbenicillin sodium, or contained 250 mg/l cefotaxime alone. As the medium-solidifier, 4 g/l Gelrite was used. The drug resistant calli grown on the medium was subjected to the X-Gluc treatment and expression of the GUS gene was checked as described above.

(6) Results

The results of the transient expression of the GUS gene after the heat

treatment and/or the centrifugation, and after the co-culturing with LBA4404(pIG121Hm) and LBA4404(pNB131) are shown in Tables 1 and 2. By carrying out the heat treatment or centrifugation treatment, the area in which GUS was expressed was clearly larger than the non-treated group, so that gene transfer occurred at a higher frequency. Further, by combining the heat treatment and the centrifugation treatment, the frequency was further increased.

The results of selection of the transformed calli obtained by culturing the rice immature embryos on the medium containing the selection drugs, after the co-culturing with *Agrobacterium* are shown in Tables 3, 4 and 5. The efficiency of obtaining transformed calli which were resistant to drug and which sowed uniform expression of GUS gene was prominently increased by carrying out the heat treatment or the centrifugation treatment. Further, by combining the heat treatment and the centrifugation treatment, the efficiency of transformation was higher than in the cases where only one of these treatments was performed (Tables 3, 4 and 5). Thus, it was proved that by subjecting rice embryo to combination of the heat treatment and centrifugation treatment, efficiency of transformation was further promoted when compared with the cases where only one of these treatments was performed.

Further, it was confirmed that in cases where the efficiency of gene transfer is low by the centrifugation treatment alone due to the variety or the like, the efficiency of gene transfer was prominently increased by co-employing the heat treatment. Further, it was also confirmed that by setting the temperature of the centrifuge at about 40°C when the centrifugation is carried out, the centrifugation treatment and the heat treatment may be carried out simultaneously, and similar effect as the above-described combination of the treatments is obtained.

Hiei et al. (1994 (Reference (13))) reported that transformation may be attained with a relatively high efficiency using calli of rice. Aldemita RR et al.

1996 (Reference (1))) reported a case of transformation using rice immature embryo. To more effectively and more stably carry out these transformation methods, the above-described combined treatment method is very effective. Especially, although the quality of immature embryo is likely varied depending on the environment of culturing so that it is not easy to always obtain immature embryo suited for transformation, it may be possible to keep high efficiency of transformation by subjecting the immature embryo to the combined treatments. Hiei et al. (1994) (Reference (13)) showed that a super-binary vector having a high transformation ability promotes the efficiency of transformation of rice. According to Aldemita RR et al. 1996 (Reference (1))), transformants were obtained only in the test using LBA4404(pTOK233) containing a super-binary vector. By the combined treatment method according to the present invention, even when an ordinary binary vector is used, a high efficiency of transformation is attained, which is comparable to or even higher than that attained in the transformation using a super-binary vector. Further, by employing both the super-binary vector and the combined treatment method, the efficiency may be even more promoted. Still further, it is expected that transformants may be obtained by employing the combined treatment method for the varieties with which a transformant has not hitherto been obtained.

Table 1 Heat/Centrifugation Treatments and Transient Expression of GUS Gene in Scutella of Immature Embryos (Variety: Asanohikari)

Treatment Temperature (Treatment Time)	Centrifugal Acceleration (Treatment Time)	Number of Sample Immature Embryos	Number of Immature Embryos						
			Percentage of GUS-Expressed Area in Surface of Scutellum (%)						
			0	0-1	1-10	10-20	20-50	50-80	80-100
-	-	20	3	8	8	1	0	0	0
46°C (5 min)	-	20	1	6	7	4	2	0	0
-	20,000G (30 min)	20	0	1	4	7	7	1	0
46°C (5 min)	20,000G (30 min)	20	0	0	0	2	9	8	1

Sample Strain: LBA4404(pIG121Hm); Duration of Co-culturing: 5 days

Table 2 Heat/Centrifugation Treatments and Transient Expression of GUS Gene in Scutella of Immature Embryos (Variety: Asanohikari)

Treatment Temperature (Treatment Time)	Centrifugal Acceleration (Treatment Time)	Number of Sample Immature Embryos	Number of Immature Embryos						
			Percentage of GUS-Expressed Area in Surface of Scutellum (%)						
			0	0-1	1-10	10-20	20-50	50-80	80-100
-	-	20	3	13	4	0	0	0	0
46°C (5 min)	-	20	0	0	10	7	3	0	0
-	20,000G (30 min)	20	0	0	3	9	8	0	0
46°C (5 min)	20,000G (30 min)	20	0	0	0	3	14	3	0

Sample Strain: LBA4404(pNB131); Duration of Co-culturing: 6 days

Table 3 Heat/Centrifugation Treatments and Efficiency of Selection of Transformed Calli (Variety: Asanohikari)

Treatment Temperature (Treatment Time)	Centrifugal Acceleration (Treatment Time)	Number of Sample Sections of Immature Embryo (A)	Number of Hm-resistant GUS-Positive Calli (B)	B/A(%)
-	-	50	6	12.0
46°C (5 min)	-	51	15	29.4
-	20,000G (30 min)	51	29	56.9
46°C (5 min)	20,000G (30 min)	46	29	63.0

Sample Strain: LBA4404(pIG121Hm); Duration of Co-culturing: 5 days, Hm: 100 mg/l hygromycin

Table 4 Heat/Centrifugation Treatments and Efficiency of Selection of Transformed Calli (Variety: Asanohikari)

Treatment Temperature (Treatment Time)	Centrifugal Acceleration (Treatment Time)	Number of Sample Sections of Immature Embryo (A)	Number of Hm-resistant GUS-Positive Calli (B)	B/A(%)
-	-	60	7	11.7
46°C (5 min)	-	60	9	15.0
-	20,000G (1min)	60	48	80.0
-	20,000G (60 min)	60	48	80.0
46°C, 5 min	20,000G (1 min)	60	51	85.0
46°C, 5 min	20,000G (60 min)	60	51	85.0

Sample Strain: LBA4404(pIG121Hm); Duration of Co-culturing: 6 days, Hm: 100 mg/l hygromycin

Table 5 Heat/Centrifugation Treatments and Efficiency of Selection of Transformed Calli (Variety: Asanohikari)

Treatment Temperature (Treatment Time)	Centrifugal Acceleration (Treatment Time)	Number of Sample Sections of Immature Embryo (A)	Number of Hm-resistant GUS-Positive Calli (B)	B/A(%)
-	-	62	18	29.0
46°C (5 min)	-	64	32	52.5
-	20,000G (30 min)	60	39	65.0
46°C, 5 min	20,000G (30 min)	60	41	68.3

Sample Strain: LBA4404(pNB131); Duration of Co-culturing: 6 days, PPT: 10 mg/l phosphinothricin

Example 2

Immature embryos of maize with a size of about 1.2 mm (variety: A188, obtained from National Institute of Agrobiological Resources, The Ministry of Agriculture, Forestry and Fisheries) were aseptically collected and placed in a tube containing 2 ml of LS-inf liquid medium. After washing the embryos once with the same medium, 2.0 ml of fresh medium was added. Heat treatment was carried out by immersing the tube in a water bath at 46°C for 3 minutes. Centrifugation

treatment was performed by centrifuging the tube with a cooling centrifuge at 20 KG, at 4°C for 30 minutes. Combined heat/centrifugation treatments were carried out by conducting the above-described heat treatment and then conducting the above-described centrifugation treatment. The control was left to stand at room temperature for the same period of time. After the treatments, the medium was removed, and 1.0 ml of a suspension of *Agrobacterium tumefaciens* LBA4404(pSB131)(Ishida et al. 1986 (Reference (20)) with a population density of about 1 x 10^9 cfu/ml in LS-inf liquid medium containing 100 μM acetosyringone rubber was added, followed by stirring the mixture with a vortex mixer for 30 seconds. After leaving the mixture to stand at room temperature for 5 minutes, the embryos were plated on LS-AS medium containing 10 μM $AgNO_3$ such that the surface of each hypocotyl contacts the medium. After co-culturing in the dark at 25°C for 3 days, an aliquot of the immature embryos was sampled and the transient expression of the GUS gene was checked by the treatment with X-gluc. The plasmid pSB131 is a super-binary vector.

The immature embryos after the co-culturing were cultured on a medium containing phsphinothricin (PPT) and 10 μM $AgNO_3$, thereby carrying out selection of the transformed cells. The calli grown on the selection medium were placed on a regeneration medium containing PPT, regeneration of transformed plants was carried out. A portion of a leaf of each regenerated plant was excised and the transient expression of the GUS gene was checked by the treatment with X-gluc as in Example 1. The above-described medium and method for culturing were in accordance with Ishida, Y. et al. 1996 (Reference (20)).

The results of transient expression of GUS gene when LBA4404(pSB131) was infected to the embryos after the treatments are shown in Table 6. All of the used immature embryos including the non-treated control, expression of GUS gene was observed. However, the area in which the GUS was expressed was larger in the

embryos subjected to heat treatment or combination of the heat treatment and centrifugation treatment than in the control. Especially, in the group subjected to the combination of the heat treatment and centrifugation treatment, the number of the embryos which showed expression of GUS gene in large area in the surface of the scutellum of each embryo was the largest.

The results of transformation of the immature embryos infected with LBA4404(pSB131) are shown in Table 7. From the control immature embryos which were not subjected to the heat treatment, transformed plants were obtained at a efficiency of 10.7%. On the other hand, the efficiency of transformation of the immature embryos subjected to the centrifugation treatment at 20KG at 4°C for 30 minutes was 13.3%, so that the efficiency was higher than that of the control group. The efficiency of the transformation of the embryos subjected to the heat treatment was 20%, which is about the twice that of the non-treated group. Further, the efficiency of transformation of the embryos subjected to the combined heat treatment and centrifugation treatment was 29.6% which was about three times that of the control group.

From the results described above, it was proved that the transformation efficiency is promoted by subjecting the immature embryos as the starting materials to centrifugation treatment or heat treatment before the infection, the transformation efficiency is even more promoted by combining these treatments. From these, the possibility that the maize varieties (Ishida et al. 1996 (Reference (20))) other than A188, which could not be hitherto transformed by the conventional *Agrobacterium* method, may be transformed by the centrifugation treatment, was suggested.

Table 6 Influence on Efficiency of Gene Transfer by Treatments (Infected with LBA4404(pSB131))

Treatment	Sample Immature Embryo	GUS			
		+++	++	+	-
Not Treated	9	0	3	6	0
Heat	9	1	7	1	0
Centrifugation	12	0	3	9	0
Heat and Centrifugation	17	5	9	3	0

Transient expression of GUS gene in immature embryos after co-culturing

Table 7 Influence on Efficiency of Gene Transfer by Treatments (LBA4404(pSB131) was transferred)

Treatment	Number of Sample Immature Embryos	PPT-resistant callus (%)	PPT-resistant plant (%)	GUS+ plant (%)
Not Treated	28	9 (32.1)	9 (32.1)	3 (10.7)
Heat	30	18 (60.0)	15 (50.0)	6 (20.0)
Centrifugation	30	14 (46.6)	9 (30.0)	4 (13.3)
Heat and Centrifugation	27	23 (85.2)	20 (74.1)	8 (29.6)

Both the number of calli and number of plants do not include clones.

Example 3

Mature seeds of creeping bent grass (*Agrostis palustris* cv. *Pencross*, Yukijirushi Shubyo Co., Ltd.) were sterilized and placed on a medium (TG2 medium) containing MS inorganic salts, MS vitamins, 4 mg/l dicamba, 0.5 mg/l 6BA, 0.7 g/l proline, 0.5 g/l MES, 20 g/l sucrose and 3 g/l gelrite (pH 5.8), followed by culturing the seeds at 25°C in the dark. The derived calli were subcultured on the medium having the same composition to grow embryogenic calli. The obtained embryogenic calli were transferred to liquid medium (TG2L) which had the same composition as TG2 except that it did not contain gelrite, and cultured under shaking at 25°C in the dark to obtain cells of suspension culture. The cells of suspension

culture on 3 to 4 days after the subculture were placed in a tube containing 2 ml of TG2L medium. After once washing the calli with the same medium, 2 ml of fresh medium was added. The tube was immersed in a water bath at 46°C for 5 minutes. After removing the medium and adding the same fresh liquid medium, the resultant was centrifuged at 5000 rpm at 4°C for 10 minutes. The control was left to stand at room temperature. The medium was removed and 1.0 ml of a suspension of *Agrobacterium tumefaciens* LBA4404(pTOK233) (described above) in TG2-inf medium (the same composition as TG2 medium except that proline, MES and gelrite are removed and 48.46 g/l sucrose, and 36.04 g/l glucose were added) at a population density of about 1 x 10^9 cfu/ml was added, followed by stirring the resulting mixture for 30 seconds by a vortex mixer. After leaving the calli to stand at room temperature for 5 minutes, the calli were placed on a medium (TG2-AS medium) which was the TG2L medium supplemented with 10 g/l glucose, 100 μM acetosyringone, 4 g/l type I agarose (pH5.8), and cultured at 25°C for 3 days i the dark. The cells were then washed three times with TG2L medium containing 250 mg/l cefotaxime and carbenicillin. The cells were then suspended in the same buffer and cultured under shaking at 25°C in the dark at 70 rpm. One week later, the cells were subcultured on a medium having the same composition except that 50 mg/l hygromycin was added. After culturing the cells for another week, an aliquot was sampled and treated with X-gluc to check the expression of the GUS gene.

Expression of the GUS gene in the suspended cultured cells of creeping bent grass infected with LBA4404(pTOK233) is shown in Table 8. In the control group, only one cell cluster showed expression of GUS. In contrast, in cases where the heat treatment and centrifugation treatment, about 30% of the cell clusters showed expression of GUS gene. Further, the area in which the GUS gene was expressed was larger in the cell clusters subjected to heat and centrifugation treatments than that of the control group.

The transformation of creeping bent grass was hitherto only attained by the direct transfer method, i.e., by particle gun (Zhong et al. 1993 (Reference (45)), Hartman et al. 1994 (Reference (12)), Xiao,L. et al., 1997 (Reference (43))) or by electroporation (Asano Y., 1994(Reference (3)), Asano Y. et al. 1998 (Reference (4))), and successful transformation by *Agrobacterium* method has not been reported. Assuming that the cause of the difficulty in transformation of creeping bent grass by *Agrobacterium* method is the low efficiency of the gene transfer in the known methods as can be seen from this Example, the possibility to obtain a transformed plant by the combined heat and centrifugation treatments according to the present invention was suggested.

Table 8 Effect of Heat and Centrifugation Treatments on Efficiency of Gene Transfer into Suspended Cultured Cells of Creeping Bent Grass

Treatment	Number of Cell Clusters		
	Total Number	GUS+	GUS+ (%)
Heat and Centrifugation Treatment	79	23	29.1
Control	101	1	1.0

Expression of GUS gene was checked two weeks after co-culturing

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CLAIMS

1. A method for promoting efficiency of gene transfer into plant cells by a bacterium belonging to genus *Agrobacterium*, comprising heating and centrifuging said plant cells or plant tissue.

2. The method according to claim 1, wherein said gene transfer is carried out after heating and centrifuging said plant cells or plant tissue.

3. The method according to claim 1 or 2, wherein the heat treatment is carried out at a temperature of 33°C to 60°C.

4. The method according to claim 3, wherein the heat treatment is carried out at a temperature of 35°C to 55°C.

5. The method according to claim 4, wherein the heat treatment is carried out at a temperature of 37°C to 52°C.

6. The method according to any one of claims 1 to5, wherein the heat treatment is carried out for 5 seconds to 24 hours.

7. The method according to claim 1 or 2, wherein the heat treatment is carried out at a temperature of 37°C to 52°C for 1 minute to 24 hours.

8. The method according to any one of claims 1to 7, wherein the centrifugation is carried out under a centrifugal acceleration of 100G to 250,000G.

9. The method according to claim 8, wherein said centrifugation is carried out under a centrifugal acceleration of 500G to 200,000G.

10. The method according to claim 9, wherein said centrifugation is carried out under a centrifugal acceleration of 1000G to 150,000G.

11. The method according to any one of claims 1 to 10, wherein said centrifugation is carried out for 1 second to 4 hours.

12. A method for preparing a plant characterized by using the method according to claim 1 to 11.

13. Plant cells, plant tissue or plant prepared by the method according to claims 1

to 12.

14. The method according to any one of claims 1 to 11, wherein said plant cells or plant tissue used are(is) originated from an angiosperm.

15. A method for preparing an angiosperm characterized by using the method according to claim 14.

16. Angiosperm cells, angiosperm tissue or angiosperm prepared by the method according to claim 14 or 15.

17. The method according to claim 14, wherein said plant cells or plant tissue used are(is) originated from a monocotyledon.

18. A method for preparing a monocotyledon characterized by using the method according to claim 17.

19. The monocotyledon cells, monocotyledon tissue or monocotyledon prepared by the method according to claim 17 or 18.

20. The method according to claim 17, wherein said plant cells or plant tissue are(is) originated from a plant belonging to family Gramineae.

21. A method for preparing a plant belonging to family Gramineae characterized by using the method according to claim 20.

22. The cells of the plant belonging to family Gramineae, the tissue of the plant belonging to family Gramineae, or the plant belonging to family Gramineae prepared by the method according to claim 20 or 21.

23. The method according to claim 20, wherein said plant cells or plant tissue are(is) of rice or maize.

24. A method for preparing rice or maize characterized by using the method according to claim 23.

25. Rice cells, rice tissue, rice, maize cells, maize tissue or maize prepared by the method according to claim 23 or 24.

ABSTRACT OF THE DISCLOSURE

A method for gene transfer by which higher efficiency for gene transfer than that by the conventional *Agrobacterium* method may be attained simply and without injuring the tissue is disclosed. According to the method of the present invention, the efficiency of gene transfer into plant cells by a bacterium belonging to genus *Agrobacterium* is promoted by accompanying heat treatment and centrifugation treatment of the plant cells or plant tissue.

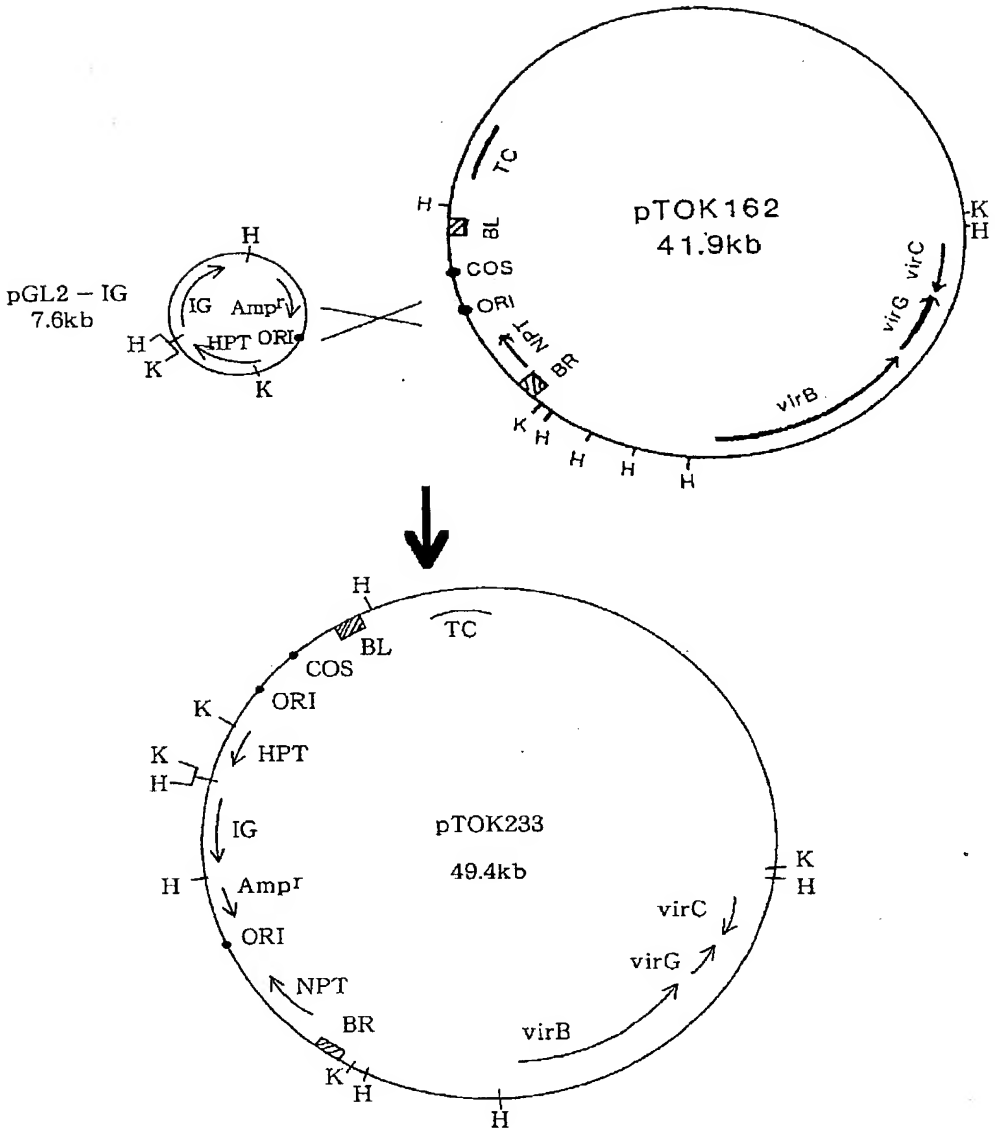
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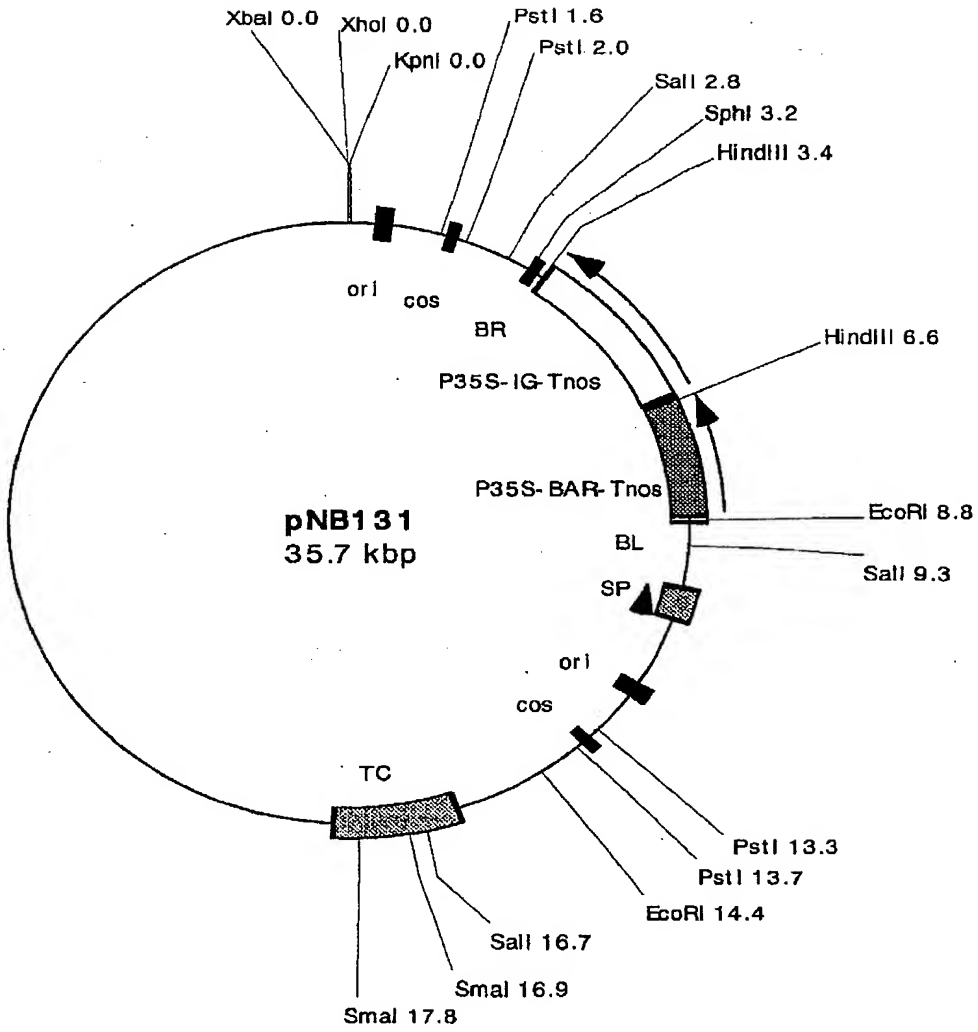
Fig. 1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ori
cos
BR
P35S-IG-Tnos
HindIII 6.6
P35S-BAR-Tnos
EcoRI 8.8
pNB131
35.7 kbp
BL
SalI 9.3
SP
ori
cos
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Fig. 2

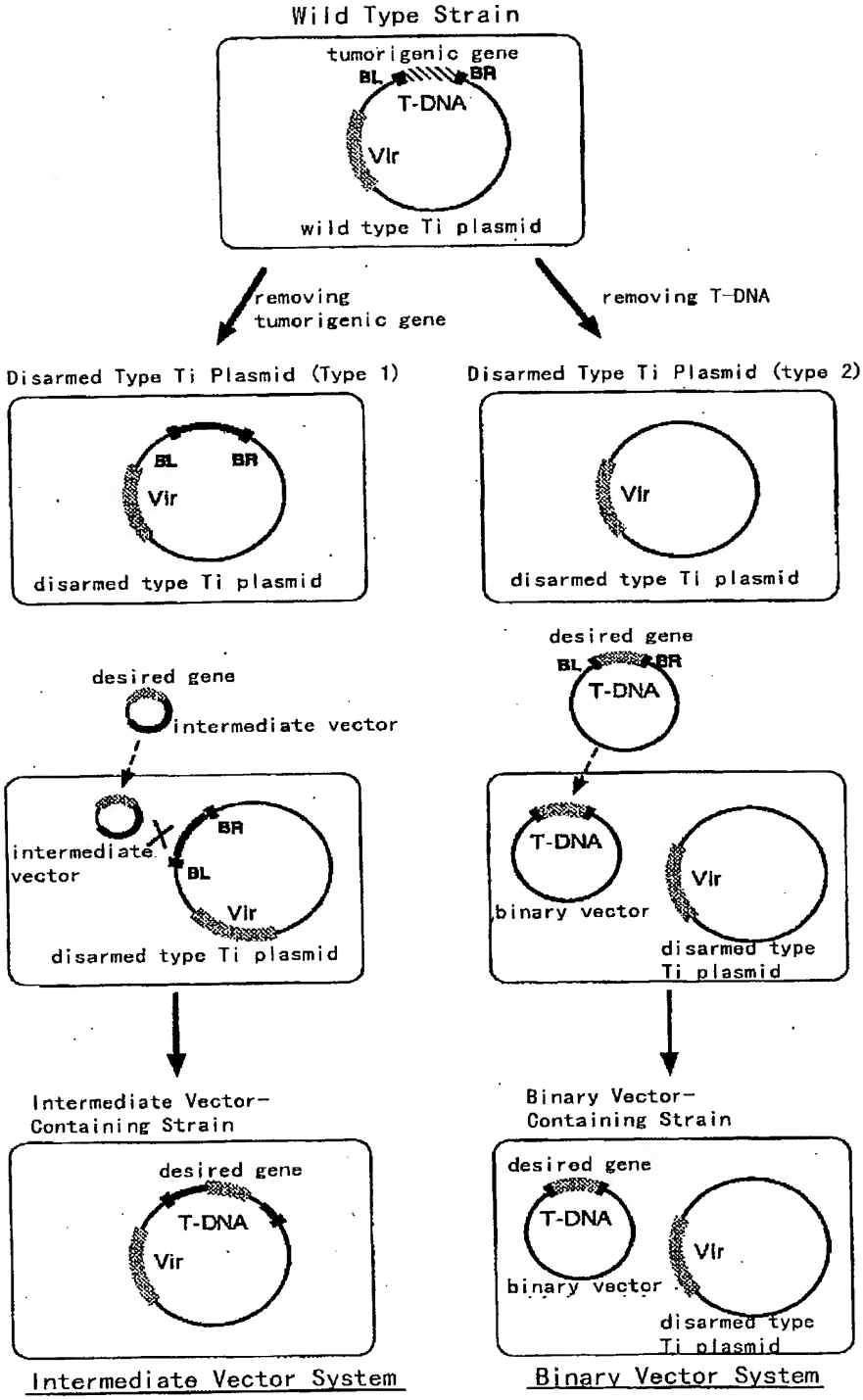
Wild Type Strain
tumorigenic gene
BL
BR
T-DNA
Vir
wild type Ti plasmid
removing tumorigenic gene
removing T-DNA
Disarmed Type Ti Plasmid (Type 1)
BL
BR
Vir
disarmed type Ti plasmid
Disarmed Type Ti Plasmid (type 2)
Vir
disarmed type Ti plasmid
desired gene
intermediate vector
intermediate vector
BR
BL
Vir
disarmed type Ti plasmid
desired gene
BL
BR
T-DNA
T-DNA
binary vector
Vir
disarmed type Ti plasmid
Intermediate Vector-Containing Strain
desired gene
T-DNA
Vir
Intermediate Vector System
Binary Vector-Containing Strain
desired gene
T-DNA
binary vector
Vir
disarmed type Ti plasmid
Binary Vector System

Fig. 3

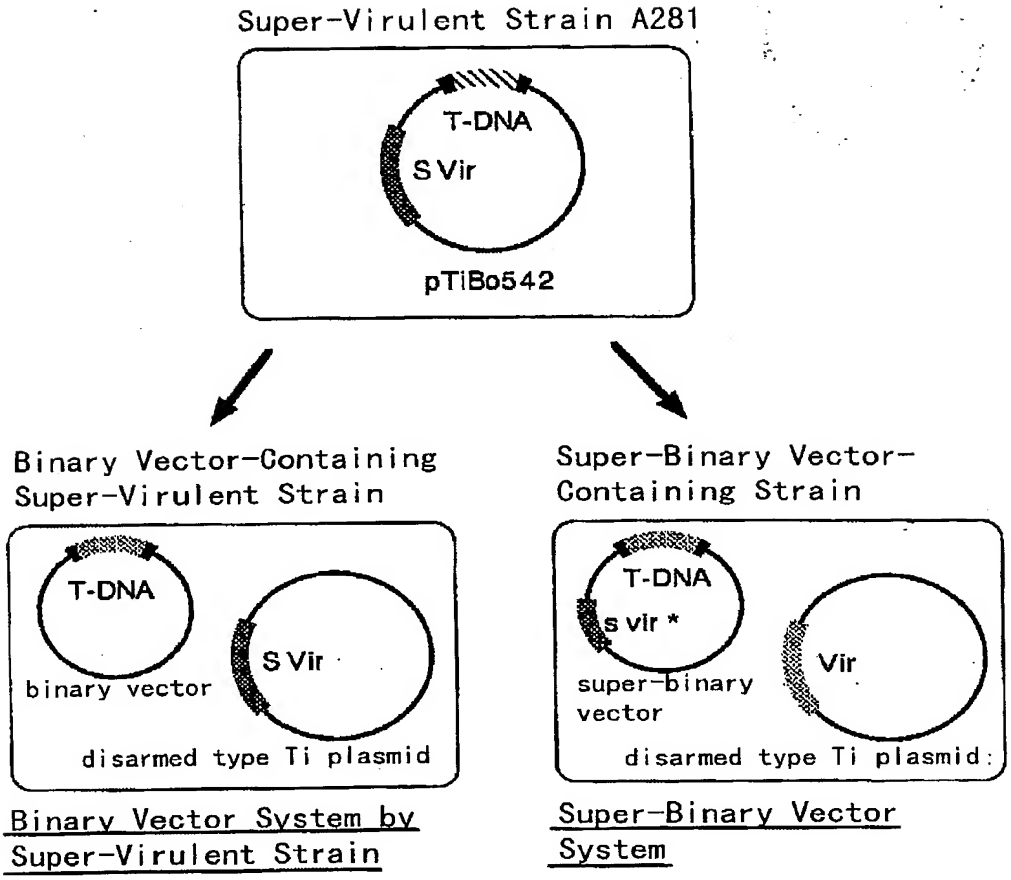
Super-Virulent Strain A281
T-DNA
S Vir
pTiBo542
Binary Vector-Containing Super-Virulent Strain
T-DNA
binary vector
S Vir
disarmed type Ti plasmid
Binary Vector System by Super-Virulent Strain
Super-Binary Vector-Containing Strain
T-DNA
s vir *
super-binary vector
Vir
disarmed type Ti plasmid
Super-Binary Vector System

Fig. 4

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COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT AND DESIGN APPLICATIONS

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated next to my name; that I verily believe that I am the original, first and sole inventor (if only one inventor is namedbelow) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:" Method for Promoting Efficiency of Gene Transfer into Plant Cells"

the specification of which is attached hereto. If not attached hereto,
the specification was filed on _____ as
United States Application Number _____;
and amended on _____ (if applicable) and/or
the specification was filed on 08/03/00 as PCT
International Application Number PCT/JP00/05214; and was
amended under PCT Article 19 on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I do not know and do not believe the same was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this applicationin any country foreign to the United States of America on an application filed by me or my legal representative or assigns more than twelve months (six months for designs) prior to this application, and that no application for patent or inventor's certificate on this invention has been filed in any country foreign to the United States of America prior to this application by me or my legal representatives or assigns, except as follows.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed
158026/99 (Number)	Japan (Country)	06/04/99 (Month/Day/Year Filed)	No
_____ (Number)	_____ (Country)	_____ (Month/Day/Year Filed)	

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional applications(s) listed below.

_____ (Application Number) _____ (Filing Date)

_____ (Application Number) _____ (Filing Date)

All Foreign Applications, if any, for any Patent or Inventor's Certificate Filed More than 12 Months (6 Months for Designs) Prior to the Filing Date of This Application:

Country	Application Number	Date of Filing (Month/Day/Year)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States and/or PCT application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States and/or PCT application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to the patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

(Application Number)	(Filing Date)	(Status - patented, pending, abandoned)
(Application Number)	(Filing Date)	(Status - patented, pending, abandoned)

10/059695 - 052102

Attorney Docket No.

I hereby appoint the following attorneys to prosecute this application and/or an international application based on this application and to transact all business in the Patent and Trademark Office connected therewith and in connection with the resulting patent based on instructions received from the entity who first sent the application papers to the attorneys identified below, unless the inventor(s) or assignee provides said attorneys witha written notice to the contrary:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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GIVEN NAME/FAMILY NAME	INVENTOR'S SIGNATURE	DATE*
Residence (City, State & Country)		CITIZENSHIP
POST OFFICE ADDRESS (Complete Street Address including City, State & Country)		

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